Chemistry and Physics of Lipids, 35 (1984) 363–370
Elsevier Scientific Publishers Ireland Ltd.

INFLUENCE OF CHOLESTEROL ESTERS OF VARYING UNSATURATION ON THE POLYMORPHIC PHASE PREFERENCES OF EGG PHOSPHATIDYLETHANOLAMINE

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Received May 3rd, 1984 revision received July 6th, 1984
accepted July 6th, 1984

Previous work has shown that cholesterol esters are relatively insoluble (<5 mol%) in bilayer phosphatidylcholine and sphingomyelin systems, but exhibit greater solubility in hexagonal (H_{II}) phase anhydrous phosphatidylcholine systems obtained above 100°C. We examine here the influence of cholesterol esters of varying unsaturation on fully hydrated egg phosphatidylethanolamine systems, which undergo a bilayer to hexagonal (H_{II}) phase transition as the temperature is increased through T_{BH} = 28°C. We show that all esters investigated (cholesteryl stearate, myristate, oleate, linoleate and linolenate) progressively decrease T_{BH} as the mole fraction of ester is increased to at least equimolar proportions. The more unsaturated species have larger effects. These results indicate that cholesterol esters are miscible in phosphatidylethanolamine dispersions and suggest a 'cone' shape of intercalated cholesterol ester. Further, the major influence of cholesterol esters on phospholipid phase structure appears to be determined by the nature of the esterified fatty acid.

Keywords: cholesterol esters; phosphatidylethanolamine; ^{31}P-NMR; freeze-fracture; hexagonal (H_{II}) phase.

Introduction

The lipid fraction of the arterial intimal wall and associated components increases remarkably with increasing age. This is manifested by various types of lipid deposition. Aged intimas contain extracellular lipid droplets, fatty streaks exhibit intracellular droplets and atherosclerotic plaques have both extra and intracellular lipid deposits [1]. The major components of these deposits are cholesterol, cholesterol ester and phospholipid, with the greatest age-related increase occurring for cholesterol esters [1]. It has been suggested [2] that the deposition and structure of the lipid deposits is dependent on the physical properties of the component lipid and that insight into potential mechanisms of lesion regression may depend on a detailed understanding of such factors.

The structure and physical properties of cholesterol and cholesterol/phosphatidylcholine systems have been extensively investigated [3,4]. However, the physical chemistry of lipid systems containing cholesterol esters has received less detailed

attention. It has been shown that cholesterol esters are relatively insoluble in bilayer phosphatidylcholine-water systems (up to 5 mol% [5]). Further studies of the phase preferences of cholesteryl linolenate-phosphatidylcholine systems in the absence [6] and presence [5] of water have been performed which were subsequently extended to include cholesterol as a further component [7]. These latter studies [7] reveal that lipid compositions (in the presence of excess water) corresponding to aged intima, as well as fatty streaks and plaques, give rise to two or three phase systems which appear to correspond to lipid structures observed in fatty streaks and atherosclerotic plaques.

In this work we examine the influence of cholesterol esters of varying unsaturation on the polymorphic phase preferences of (egg yolk) phosphatidylethanolamine (in the presence of excess water) which undergoes a transition from the bilayer to the hexagonal H_{II} organization as the temperature is increased through 30°C [8]. Previous X-ray studies [6] have shown that cholesteryl linolenate incorporates more readily into (anhydrous) egg phosphatidylcholine at high (>100°C) temperatures where H_{II} organization is favoured. We show that unsaturated cholesterol esters appear to incorporate readily into fully hydrated egg phosphatidylethanolamine systems and that their presence is manifested by a reduction in the bilayer to hexagonal (H_{II}) phase transition temperature T_{BH}. This ability of cholesterol esters may be related to the mechanisms by which they segregate into lipid-rich domains.

Materials and Methods

Egg yolk phosphatidylethanolamine was isolated from hen egg yolk employing a Waters Prep-500 LC apparatus according to established procedures [9], and was greater than 99% pure as established by two-dimensional thin-layer chromatography (TLC). Cholesterol esters were obtained from Sigma (St. Louis) and were found to be >99% of the stated molecular species as indicated by gas-liquid chromatography (GLC).

Samples were prepared from mixtures of egg phosphatidylethanolamine with a given cholesterol ester in chloroform (100 mg phospholipid) and the chloroform was subsequently removed by evaporation under a stream of nitrogen and storage under vacuum for 1 h. The lipid mixture was then hydrated in an aqueous buffer containing 10 mM HEPES (pH = 7.0), 100 mM NaCl, 2 mM EDTA and 10% (v./v.) D_2O by vortexing extensively at room temperature.

81.0 MHz ^{31}P nuclear magnetic resonance (NMR) spectra were obtained employing a Bruker WP 200 Fourier Transform spectrometer equipped with temperature control and proton decoupling facilities. Free induction decays were accumulated using an 11 μs 90° radio frequency pulse, 0.8 s interpulse delay and a 25 KHz sweep width from up to 1000 transients. An exponential filter corresponding to a 50 Hz line-broadening was applied prior to Fourier transformation. All samples were checked for decomposition products following an experimental sequence. No such

products (lyso phosphatidylethanolamine, fatty acid) were visible employing TLC techniques, indicating such degradation was <1%. Freeze-fracture electron microscopy was performed employing a Balzers BAF 400 apparatus utilizing standard techniques. The replicas were visualized employing a Phillips 400 electron microscope.

Results and Discussion

The phase preferences of egg phosphatidylethanolamine in pure systems as well as the systems containing various cholesterol esters were ascertained primarily employing ^{31}P-NMR techniques (for a review of ^{31}P-NMR techniques as applied to membranes, see Ref. 10). Briefly, phospholipids in large (diameter >2000Å) bilayer structures exhibit broad 'solid state' ^{31}P-NMR spectra with a low field shoulder and high field peak separated by approx. 40 ppm. Hexagonal (H_{II}) phase phospholipids, on the other hand, give rise to spectra with reversed asymmetry which are a factor of two narrower. Finally, phospholipids in small lamellar structures, or other phases which allow isotropic motional averaging via lateral diffusion processes (e.g. inverted micelle [11] and cubic [12] configurations) evidence narrow, symmetric ^{31}P-NMR responses. The ^{31}P-NMR behaviour reflecting the phase behaviour of fully hydrated egg yolk phosphatidylethanolamine is illustrated in the first column of Fig. 1,

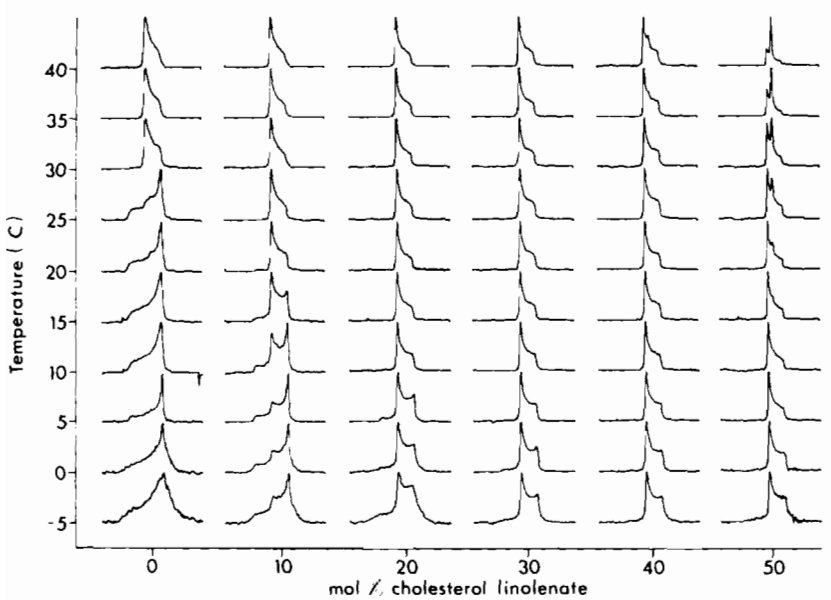

Fig. 1. 81.0 MHz ^{31}P-NMR spectra of aqueous dispersions of egg yolk phosphatidylethanolamine obtained as a function of temperature and cholesteryl linolenate content.

where a progression from a bilayer ^{31}P-NMR lineshape (below 25°C) to a hexagonal H_{II} lineshape (above 30°C) is observed as the temperature is increased. This is in accord with previously published results [8]. The influence of increasing amounts of cholesteryl linolenate on the phase preferences of egg phosphatidylethanolamine are also illustrated in Fig. 1. Two effects are apparent as the cholesteryl linolenate content is increased, namely, a decrease in the bilayer to hexagonal (H_{II}) transition temperature T_{BH} and an increase in the temperature range over which the transition takes place, corresponding to a decrease in cooperativity. At equimolar levels of cholesteryl linolenate and higher temperatures ($\geq$25°C) a narrow ^{31}P-NMR spectral component is also observable, indicating the presence of phospholipid in small structures allowing isotropic motion. Such a feature may correspond to phosphatidylethanolamine which is effectively solubilized by the liquid crystalline cholesteryl linolenate.

The ability of cholesteryl linolenate to promote H_{II} phase structure in egg phosphatidylethanolamine systems as indicated by ^{31}P-NMR is also supported by the freeze-fracture results indicated in Fig. 2. In the absence of the ester, egg phosphatidylethanolamine (quenched from 20°C) reveals the flat fracture faces typical of bilayer phospholipids where the ridges correspond to jumps of the fracture plane between adjacent bilayers. In contrast, the presence of equimolar cholesteryl linolenate results in the corrugated pattern generated as the fracture plane cleaves between the hexagonally packed lipid cylinders [13]. This is diagnostic for the H_{II} phase, and thus fully supports the ^{31}P-NMR results of Fig. 1. In this regard, it should be noted that although the ^{31}P-NMR technique for identification of lipid phase structure has been criticized [14,15], it is in fact most reliable as demonstrated by the very close correspondence between X-ray and ^{31}P-NMR studies from a number of laboratories [16–18].

It is of interest to examine the influence of the degree of unsaturation of the cholesterol ester on the ability to promote H_{II} phase structures. Studies were therefore performed on mixtures of egg phosphatidylethanolamine with cholesteryl stearate, myristate, oleate and linoleate and the results obtained are summarized in Fig. 3. A basic feature observed is that as the cholesterol ester unsaturation is increased, the mixtures displayed an enhanced proclivity for the H_{II} arrangement. Thus the midpoint of the bilayer H_{II} transition decreases from 28°C [8] in the absence of ester to 20°C, 15°C, 10°C and 5°C for equimolar concentrations of cholesteryl stearate, myristate, oleate and linoleate respectively. It may also be observed that in all cases addition of the cholesterol esters progressively reduces T_{BH} with little or no evidence of saturation at higher (equimolar) cholesterol ester contents. This suggests that egg phosphatidylethanolamine-cholesterol ester systems exist as one phase systems, at least up to equimolar levels of the ester. This is in accord with the observation that no evidence of gross phase separation of cholesterol esters in equimolar egg phosphatidylethanolamine-cholesteryl linoleate systems could be seen employing phase contrast microscopy (results not shown). Such separation would be expected to result in observation of small, highly refractile droplets.

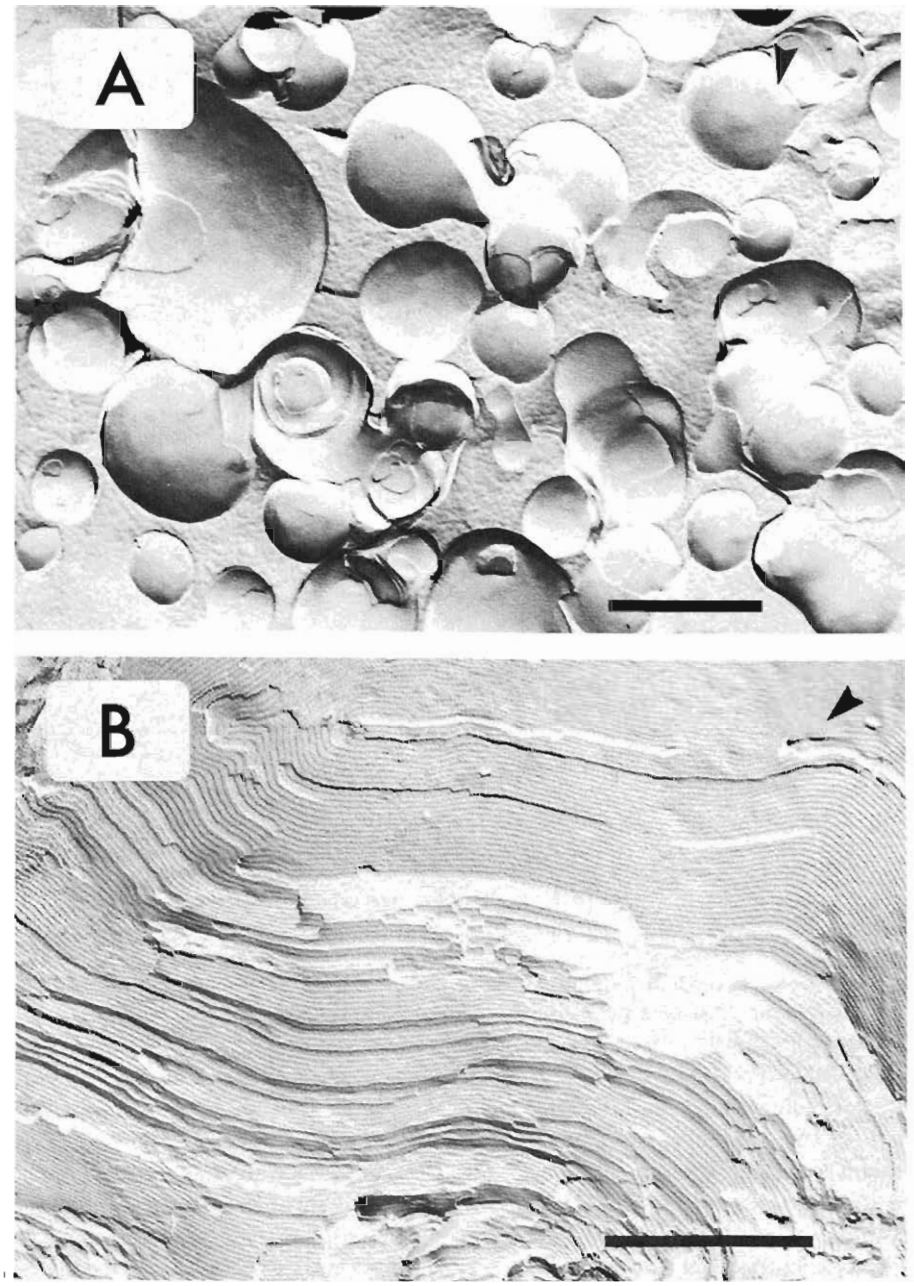

Fig. 2. Freeze-fracture electron micrographs of (a) egg yolk phosphatidylethanolamine and (b) egg yolk phosphatidylethanolamine/cholesteryl linolenate (1 : 1). The samples contained 25% (v/v) glycerol to prevent freeze damage and were quenched from 20°C. The black bar denotes 400 nm and the direction of shadowing is indicated by the arrowhead.

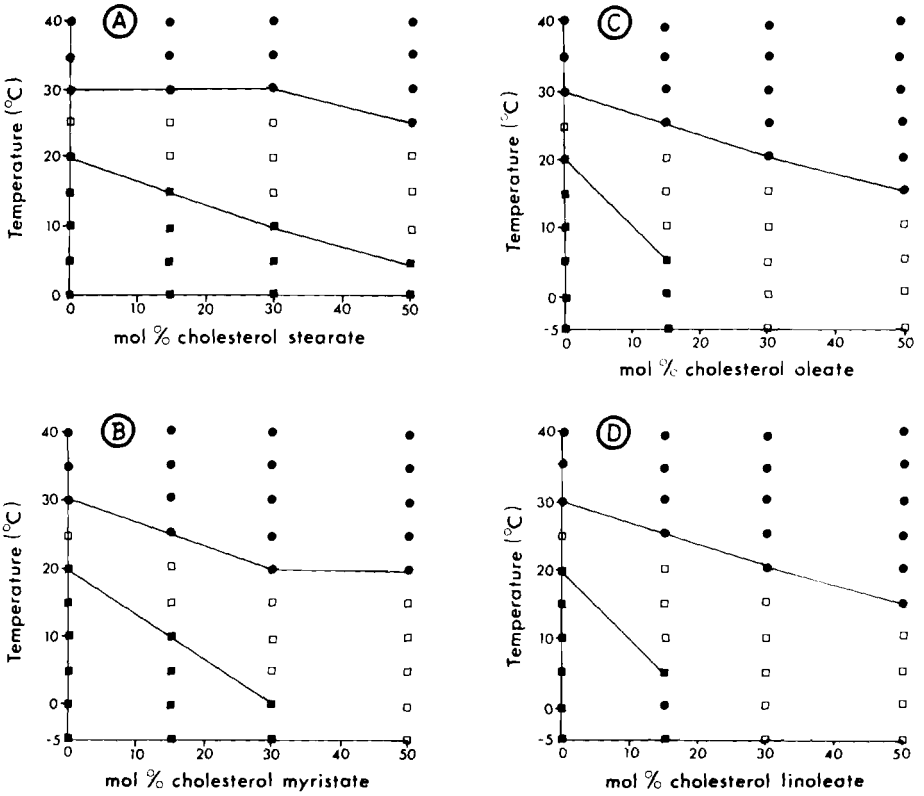

Fig. 3. Polymorphic phase characteristics of egg yolk phosphatidylethanolamine in the presence of varying amounts of (a) cholesteryl stearate; (b) cholesteryl myristate; (c) cholesteryl oleate; (d) cholesteryl linoleate. The data were collected by a ^{31}P-NMR analysis (see Fig. 1). ○, hexagonal H_{II} phase lipid; ■, bilayer phase; □, situations where both the H_{II} phase bilayer components coexist.

The predilection of a particular phospholipid species for bilayer or H_{II} structure has been previously rationalized on the basis of their molecular 'shapes' [8]. According to this hypothesis, lipids adopting the bilayer arrangement exhibit a net cylindrical shape, whereas lipids preferring the hexagonal phase exhibit a 'cone' shape (where the polar headgroup subtends a smaller cross-sectional area than the acyl chains) compatible with that structure. The ability of the cholesterol esters to destabilize bilayer structure may then be ascribed to an ability to increase the disorder in the phospholipid acyl chain region (leading to increased cone shape character) or to a net 'cone' shape of the cholesterol ester itself when intercalated into the phosphatidylethanolamine matrix. It is of interest, in this regard, to compare the influence of the cholesterol esters on egg phosphatidylethanolamine polymorphism with that of (unesterified) cholesterol, which does not affect the T_{BH} of this

phospholipid at equimolar concentrations [8]. A case could be made that the conformation of the cholesterol moiety with respect to the phosphatidylethanolamine polar headgroup is unaffected by the esterification of an acyl chain, and that the major influence on the polymorphic preferences arises due to the presence and properties of the fatty acid residue. This would be consistent with the ability of (unsaturated) fatty acids to induce H_{II} organization [19,20] as well as ^{2}H-NMR studies indicating that the acyl chain of esterified cholesterol is folded back (with the ester bond closest to the polar region) in (bilayer) mixtures with sphingomyelin [21].

The relation of these results to lipid droplet formation or the development of atherosclerotic plaques in vivo is clearly tenuous, particularly given the fact that phosphatidylethanolamine is a minority component of the phospholipid in the atherosclerotic lesion [2]. However, we do believe that the studies presented here have potentially important ramifications. In particular, our results indicate that cholesterol esters can encourage H_{II} phase formation in combinations with other phospholipid, and that this ability becomes more pronounced as the acyl chain becomes more unsaturated. Lipids with similar properties [19,22] (such as unsaturated fatty acids) promote fusion processes in model and biological systems. It is clear that in order for ester derived from a membrane bilayer to be released in the form of droplets, some (reversed) fusion event must occur, and it may be that the shape of the ester molecule itself promotes such segregation.

Acknowledgements

This research was supported by the British Columbia Heart Foundation. PRC is an MRC Scientist.

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